Proc. Nat. Acad. Sci. USA
Vol. 71, No. 2, pp. 359–362, February 1974

Differential Interaction of Cholesterol with Phosphatidylcholine on the Inner and Outer Surfaces of Lipid Bilayer Vesicles

(chemical shift/inner and outer monolayers of vesicle bilayers/cholesterol–phospholipid interaction)

CHING-HSIEN HUANG, JAMES P. SIPE, S. T. CHOW, AND R. BRUCE MARTIN

The Departments of Biochemistry and Chemistry, University of Virginia, Charlottesville, Va. 22901

Communicated by Jesse W. Beams, October 1, 1973

ABSTRACT **The separate identification of the $-N^+(CH_3)_3$ groups located on the outside and inside of phospholipid bilayers observed in proton magnetic resonance spectroscopy upon addition of low concentrations of praseodymium ion was exploited to investigate the effects of incorporated cholesterol. Only about 7% of the phosphate groups on the outside of the bilayer belong to a class of strong binding or shifting sites. Upon addition of up to about 30% cholesterol to egg lecithin bilayers, no changes in chemical shift or ratio of areas of peaks due to outer and inner $-N^+(CH_3)_3$ groups appear. At about 30% incorporated cholesterol, an abrupt decrease occurs in the chemical-shift difference between $-N^+(CH_3)_3$ groups located on the outer and inner bilayer surfaces, and an abrupt increase occurs in the ratio of the areas of the two peaks. For L-α-dipalmitoyl lecithin bilayers, an abrupt change in chemical-shift difference occurring between 10 and 20% cholesterol is not accompanied by a change in the relative number of $-N^+(CH_3)_3$ groups located on the outer and inner surfaces. These results are interpreted as due to the homogeneous distribution of up to 30% cholesterol in egg lecithin bilayers. Above 30%, cholesterol is asymmetrically distributed in favor of the inner layer. In egg lecithin with a variety of polyunsaturated side chains, the side chains with the greater number of double bonds are preferentially displaced by high concentrations of cholesterol, which accounts for the increase in the ratio of outer to inner $-N^+(CH_3)_3$ groups. Such preferential displacement by cholesterol cannot occur with the saturated L-α-dipalmitoyl lecithin. It is suggested that modified phospholipid vesicles of low radii of curvature may provide high concentrations of "active sites" present in membranes.**

Proton nuclear magnetic resonance (PMR) spectroscopy has been applied extensively in recent years to elucidate the structural or conformational properties of phospholipids and biomembranes (1). Most of these studies, however, are concerned primarily with the mobility of fatty acyl chains of the bilayer (2, 3). As reported several times (4–6), paramagnetic ions such as europium (Eu^{3+}) added to the exterior of lipid vesicles shift the field position of the $-N^+(CH_3)_3$ protons of phosphatidylcholine (PC) molecules that reside on the outer surface of lipid vesicles, which are closed shell-like spheres of about 250 Å in diameter, each comprised of a single, continuous, lipid-bilayer membrane enclosing a volume of aqueous solution (7). Using these paramagnetic ions as shift markers, the head groups in the two halves of vesicle bilayers can be spectrally identified. Consequently, the application of PMR technique has now been extended to investigate the structural properties of the outer and (or) inner polar head regions of the bilayer.

The interaction of phospholipid with cholesterol in the bilayer structure has been studied recently by a variety of physical techniques (8–10). On the basis of these studies, it has been proposed that the effect of cholesterol on phospholipids in the bilayer structure is to restrict differentially the number of steric conformations accessible to the segments of the fatty acyl chains (11). However, more recent studies have shown that the 3β-OH group of cholesterol is also of crucial importance for phospholipid–sterol interaction (12, 13), and, as a consequence, interactions in the polar head regions of the bilayer should be examined further.

In this preliminary communication, we report the effects of cholesterol content on the $-N^+(CH_3)_3$ proton resonance signal of the vesicle bilayer in the presence of a paramagnetic lanthanide ion, praseodymium ion (Pr^{3+}). We chose Pr^{3+} rather than Eu^{3+} as a shift marker to study differentially the two polar head regions of the vesicle bilayer because, at a lower concentration, the Pr^{3+} ions produced at greater spectral splitting of the $-N^+(CH_3)_3$ resonance signal upon coordination with the polar head groups of the bilayer.

EXPERIMENTAL MATERIALS AND PROCEDURES

Cholesterol was obtained from Sigma Co., St. Louis, Mo., and further purified through bromination according to the method of Schwenk and Werthessen (14). L-α-Dipalmitoyl PC was purchased from Calbiochem, La Jolla, Calif. Egg PC was isolated and purified as described previously (7). Praseodymium chloride was obtained from Alfa Ventron, Beverly, Mass., and deuterium oxide (approximately 99.9 mol %) was supplied by Bio-Rad Laboratory, Richmond, Calif. PC vesicles were prepared by ultrasonic irradiation, under argon atmosphere, for 2.5 hr at 20 KHz. The sonication temperature in the preparation of egg PC vesicles was held at 2°. L-α-Dipalmitoyl PC displays a sharp transition at 41°. Sonication of the material to produce single-walled vesicles was carried out at 50°. The L-α-dipalmitoyl PC vesicle solution was constantly kept at 47°.

PMR spectra were recorded with a Perkin-Elmer R-20a NMR Spectrometer operating at 60 MHz. Probe temperature was held at 33° ± 2° for the egg PC vesicle solution, while a temperature of 47° ± 2° was maintained for the L-α-dipalmitoyl PC vesicle solution. Probe temperatures were set with a Perkin-Elmer R-202VT variable temperature controller. The probe temperature was monitored with a standard ethylene glycol sample. Spectra of the vesicle solution containing increasing amounts of Pr^{3+} were recorded, and chemi-

Abbreviations: PMR, proton nuclear magnetic resonance; PC, phosphatidylcholine; Pr^{3+}, praseodymium ion; Eu^{3+}, europium ion; P_i, lipid phosphorus.

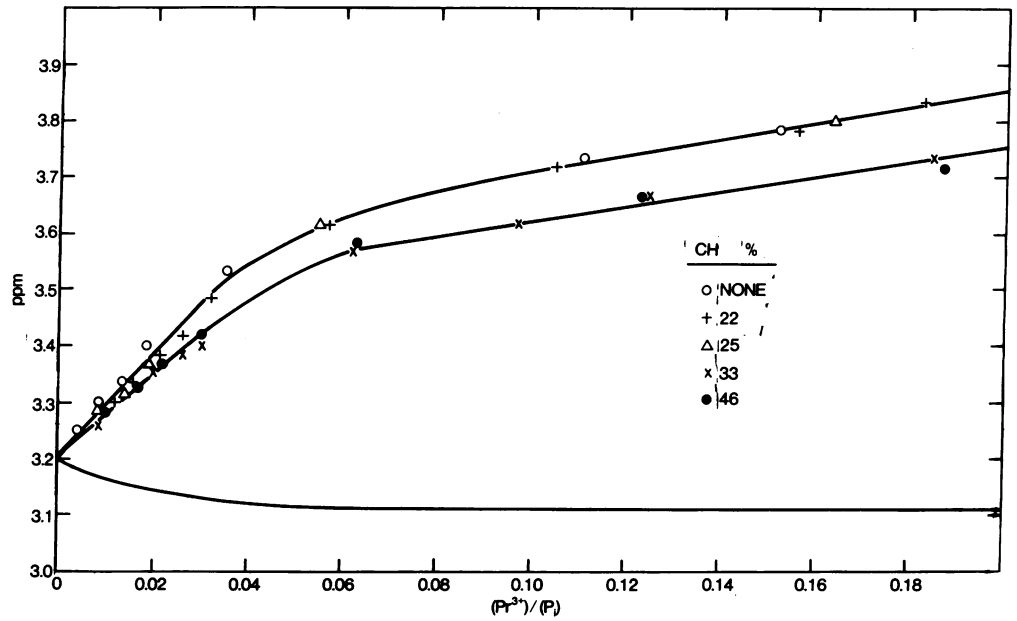

FIG. 1. Chemical shift (in ppm) downfield from internal 3-(trimethylsilyl-1-propane sulfonic acid *versus* the ratio of Pr^{3+} to egg phosphatidylcholine molar concentrations. The *two upper curves* refer to external $-N^+(CH_3)_3$ groups at the concentrations of cholesterol specified in the figure. The *lowest curve* is the average chemical shift for internal $-N^+(CH_3)_3$ groups.

cal shifts, reported in parts per million (ppm), were measured using as an internal standard the sodium salt of 3-(trimethylsilyl)-1-propane sulfonic acid. Concentrations of Pr^{3+} per mmol of lipid phosphorus, P_i, R = $[Pr^{3+}]/[P_i]$, ranged from 0.0 to 0.247, and the concentration of P_i was kept relatively constant around 55 ± 5 mM.

The total lipid concentrations of PC vesicles containing various amounts of cholesterol were determined by dry weight (7). PC concentrations are expressed in terms of P_i as determined colorimetrically according to Gomori (7). The difference between the dry weight and P_i was used to calculate the cholesterol concentration.

RESULTS AND DISCUSSION

The protons of the choline $-N^+(CH_3)_3$ groups of egg PC vesicles which show up as a single peak in the PMR spectrum at 60 MHz undergo a splitting upon addition of Pr^{3+}. The essentially unshifted smaller portion of the original peak is assigned to choline protons on the inside of the bilayer to which Pr^{3+} does not have access. The greater portion of the original peak that is shifted to lower field by addition of Pr^{3+} is assigned to external choline methyl groups. This shift of external choline methyl groups is in the opposite direction to that observed for Eu^{3+}; this difference is anticipated by comparison with other compounds (15). Only a minimal amount of broadening occurs on addition of Pr^{3+}. The ratio of integrated areas of shifted and unshifted peaks is found to be 2.18 ± 0.03 and is independent of the Pr^{3+} concentration. This value is in good agreement with the 2.16 ratio of the area of the external to the internal surface of the vesicle bilayer based on the thickness of the bilayer (about 40 Å) and the external radius (125 Å) of the spherical vesicle (7).

The magnitude of the chemical shift of the $-N^+(CH_3)_3$ signals obtained with different ratios of the Pr^{3+} and phospholipid molar concentration are given in Fig. 1. The changes in chemical shift with $(Pr^{3+})/(P_i)$ are much larger at lower ratios of $(Pr^{3+})/(P_i)$ and a smaller change occurs at the higher ratios. A minimum of two classes of binding sites with different association constants and (or) chemical shifts of Pr^{3+}-bound phospholipid is suggested by the curve. Fig. 1 also shows that the initial slope and the plateau portion of the curve intersect near 0.05 mol ratio of Pr^{3+} to P_i, indicating that only about 7% of the phospholipid groups on the outer surface of the vesicle bilayer belong to a class of strong binding or shifting sites.

The binding sites for the Pr^{3+} ions on the surface of the vesicle bilayer may be reasonably assumed to be the negatively charged phosphate moieties of choline groups embedded near the interface between the polar head region and the hydrocarbon core of the bilayer. In order to coordinate with the phosphate group, aquated Pr^{3+} ions must penetrate into the polar head region. Metal ions exchange rapidly among all available sites and produce a dipolar interaction with nearby protons resulting in a chemical shift of their resonances. Due to the rapid exchange of the Pr^{3+} ions, the observed proton chemical shifts are a weighted average of those for sites that are free and bound to lanthanide ion. The molecular packing of polar head groups cannot be flexible enough to permit every phosphate group to be approached by the aquated Pr^{3+}. Our result that only about 7% of the phosphate groups on the outer surface of the bilayer serve as strong binding sites for aquated Pr^{3+} seems reasonable.

The presence of cholesterol up to 25 mol % in the egg PC vesicles has no effect, within the precision of experimental measurements, on either the ratio of the areas of the external $-N^+(CH_3)_3$ peak to the internal $-N^+(CH_3)_3$ peak (Table 1) or the chemical shift caused by Pr^{3+} (Fig. 1). We can conclude from these results that (i) the incorporation of small amounts of cholesterol molecules into the lipid vesicle does not affect the relative number of PC molecules in the two halves of the bilayer, and (ii) the overall *polar* group packing of the original bilayer appears to be unperturbed by the presence of cholesterol, up to about 25 mol %.

Fig. 1 shows that the changes in chemical shift of the external choline protons caused by various concentration ratios of Pr^{3+}/P_i undergo an abrupt decrease on going from 25 to 33 mol % of incorporated cholesterol. This decrease in chemical shift over the entire range of Pr^{3+} concentrations used indicates that the electronic environments of the binding sites or phosphate groups on the external surface of the vesicle bilayer have been perturbed by the newly added cholesterol. X-ray studies on phospholipid–cholesterol systems indicate that the 3β-OH group of the cholesterol molecule is exposed to the polar region of the bilayer and the molecular area of phospholipid undergoes condensation in the presence of cholesterol (16). One simple explanation is that on increasing the cholesterol content from 25 to 33 mol %, the average molecular area of egg PC in the vesicle has condensed to such a dimension that the 3β-OH group of cholesterol molecules can spatially approach the neighboring phosphate group of PC molecules, thereby perturbing the electronic environment of the binding site and resulting in a decrease in chemical shift.

The maximum number of cholesterol molecules that may be accommodated in an egg PC vesicle may be calculated to be 1224. This value, based on x-ray data of Levine and Wilkins (17) for the molecular area of (*i*) egg PC (62.7 Å^2), (*ii*) condensed egg PC (46.7 Å^2) caused by the presence of saturated concentration of cholesterol (1:1 molar ratio mixture), and (*iii*) cholesterol (35 Å^2) is obtained as follows: $(62.7 - 46.7) \times 2678/35 = 1224$, where 2678 is the number of PC molecules per vesicle (7).

The mole percent cholesterol corresponding to the calculated maximum amounts of the additives that can be accommodated in the vesicle bilayer is $1224/(1224 + 2678) = 31\%$. From this simple calculation, one may expect that below 31 mol % the incorporated cholesterol molecules are probably homogeneously partitioned into the large liposomes, from which the small vesicles are formed, and the total PC concentrations in the vesicle bilayer may be independent of added cholesterol. Above 31 mol % however, the cholesterol molecules with their smaller molecular dimensions will now be preferentially packed into the curved portions of liposomes. Consequently, the chemical compositions within the liposomes are likely to be inhomogeneous, and the distribution of cholesterol molecules in the bilayer depends on the surface curvature. Since it is most likely that small vesicles are formed from the curved portions of the large liposomes, it is thus possible to make vesicles with cholesterol content higher than 31 mol %. Because of the substitution or displacement of phosphatidyl–chloines by cholesterol molecules on the curved portion of liposomes, the PC concentrations in the small vesicles would be proportionately decreased with increasing concentration of substituted cholesterol.

Table 1 shows that the ratio of areas under peaks due to external and internal choline methyl protons increases from 2.2 to 2.9 with an increase from 25 to 33 mol % of cholesterol. This result indicates that the relative number of PC molecules in the inner monolayer of the vesicle bilayer decreases from 31 to 26% of the total PC molecules over a rather narrow range of cholesterol content. The relatively lower mole percent of PC in the inner monolayer may arise from a larger percentage substitution of PC by cholesterol in the inner monolayer. Although the mechanism of substitution is unknown, one may reasonably assume that polyunsaturated PC molecules such as (1-palmitoyl-2-arachidonoyl)-3-PC, which are more loosely packed within the bilayer due to the steric hindrance of their polyunsaturated double bonds, are displaced by cholesterol more readily in the curved bilayer. If the phospholipids with polyunsaturated double bonds are, on average, asymmetrically located in the inner half of the original curved portions of liposomes, relatively more phospholipids in the inner monolayer will be first displaced by cholesterol molecules. The small vesicles, pinched off from the curved portion of liposome bilayer by ultrasonic irradiation, will then have a relatively smaller number of $-N^+(CH_3)_3$ groups in the inner monolayer of the bilayer.

In order to ascertain that the relative decrease in number of the choline methyl groups in the inner surface is related to the presence of double bonds in the acyl chains of PC molecules, similar experiments were carried out with PC vesicles composed of L-α-dipalmitoyl PC and various mole percents of cholesterol. Since there is no double bond present at all in the acyl chain of L-α-dipalmitoyl PC molecules, there is no possibility of any kind of double bond asymmetry in the bilayer. One would thus expect a constant ratio of choline protons signal of the outer to inner surfaces of the L-α-dipalmitoyl PC vesicles upon addition of various concentrations of cholesterol. Table 1 shows, indeed, there is no change in relative peak areas of the outer to inner choline resonance at various concentrations of added cholesterol. The experimental results do not, however, rule out the possibility of having asymmetrical distribution of gauche configurations between the two monolayers of the L-α-dipalmitoyl PC vesicle. Table 1 also shows that the transition in chemical shift of the external choline protons of L-α-dipalmitoyl PC vesicles caused by Pr^{3+} ions occurs somewhere between 10 and 20 mol % of incorporated cholesterol, whereas the equivalent transition in egg PC vesicles is around 29 mol % of cholesterol. This difference may be attributed as shown below to the smaller molecular dimension of L-α-dipalmitoyl PC.

As mentioned above, the average molecular area of egg PC in the vesicle bilayer, 63 Å^2, will be condensed to 47 Å^2 as the concentration of incorporated cholesterol reaches 31 mol %, the maximum amount of cholesterol that can be accommodated in the curved bilayer without displacing PC molecules. Assuming the condensation of PC area is linearly related to the cholesterol content, it is possible to calculate the average molecular area of egg PC in the presence of various concentrations of cholesterol up to 31 mol %. Therefore, the mean

TABLE 1. *Effects of cholesterol on PMR parameters of phosphatidylcholine vesicles*

Phosphatidyl-choline	Cholesterol mol %	$\Delta\nu$* ppm	Area† external/internal
Hen egg‡	0–25	0.47	2.2
	33, 46	0.41	2.9
L-α-Dipalmitoyl§	0, 10	0.28	2.2
	20–40	0.22	2.2

* Between external and internal $-N^+(CH_3)_3$ groups at 0.05 mol ratio of Pr^{3+}/P_i.

† Ratio of areas of peaks due to external and internal $-N^+(CH_3)_3$ groups.

‡ At 33°.

§ At 47°.

molecular area with a dimension such that the electronic environment of the phosphate groups can be perturbed by neighboring cholesterol molecules, as suggested by the transition in the chemical shift, can be calculated from the corresponding cholesterol concentration. Since the transition in chemical shift occurs on going from 25 to 33 mol % of cholesterol in egg PC vesicles, a molecular dimension of 48 Å^2 may be estimated from the midpoint of the concentration range. Taking this value, 48 Å^2, and the molecular area of pure L-α-dipalmitoyl PC in the liquid crystalline state, 58 Å^2 (18), one can calculate at what concentration of cholesterol in the L-α-dipalmitoyl PC vesicle we should observe a transition in chemical shift, assuming, to the first approximation, the condensation of molecular area of L-α-dipalmitoyl PC caused by cholesterol is parallel to that of egg PC. The calculated concentration of cholesterol is 19 mol %, while the experimentally observed transition occurs somewhere between 10 and 20 mol %. This agreement strongly suggests that the decrease in chemical shift may, indeed, be coupled with the decrease in the average molecular area of PC induced by the condensing effect of cholesterol.

Due to the low radius of curvature, the usefulness of lipid vesicle bilayers as models for natural membrane systems has been questioned. The above results have been interpreted in terms of an asymmetry in composition of the two sides of egg PC vesicles and of their differential interaction with cholesterol. Extension of these conclusions suggests that lipid vesicles with low radii of curvature may be especially appropriate models for the "active site" regions of membranes. It is generally recognized that a membrane is not uniform, but that for each of its many functions only specialized areas are effective. These areas often occur in highly folded regions of the membrane with low radii of curvature. Examples are cristie in inner mitochondrial membranes and the brush borders of intestinal epithelial cells. That only a fraction of a membrane surface is effective in participating in most functions recalls the many classical studies of physical chemists on heterogeneous catalysts where only a small fraction of the surface possesses the appropriate specialized configuration to serve as an active site for catalysis of a reaction. Suitably modified small lipid vesicles may provide high concentrations of "active sites" useful in the delineation of membrane functions.

This investigation was supported by USPHS Grant GM-17452 and NSF Grant GB-29328.

1. Horwitz, A. (1972) in *Membrane Molecular Biology*, eds. Fox, C. F. & Keith, A. D. (Sinauer Associates, Stanford, Calif.), pp. 164–191.
2. Finer, E. G., Flook, A. G. & Hauser, H. (1972) *Biochim. Biophys. Acta* **260**, 59–69.
3. Horowitz, A. F., Michaelson, D. & Klein, M. P. (1973) *Biochim. Biophys. Acta* **298**, 1–7.
4. Bystrov, V. F., Dubrovina, N. I., Barsukov, L. I. & Bergelson, L. D. (1971) *Chem. Phys. Lipids* **6**, 343–350.
5. Kostelnik, R. J. & Castellano, S. M. (1972) *J. Magn. Resonance* **7**, 219–223.
6. Levine, Y. K., Lee, A. G., Birdsall, N. J. M., Metcalfe, J. C. & Robinson, J. D. (1973) *Biochim. Biophys. Acta* **291**, 592–607.
7. Huang, C. (1969) *Biochemistry* **8**, 344–352.
8. Ladbrooke, B. D., Williams, R. M. & Chapman, D. (1968) *Biochim. Biophys. Acta* **150**, 333–340.
9. Hinz, H.-J. & Sturtevant, J. M. (1972) *J. Biol. Chem.* **247**, 3697–3700.
10. Engelman, D. M. & Rothman, J. M. (1972) *J. Biol. Chem.* **247**, 3694–3697.
11. Rothman, J. E. & Engelman, D. M. (1972) *Nature New Biol.* **237**, 42–44.
12. de Kruyff, B., Demel, R. A. & van Deenen, L. L. M. (1972) *Biochim. Biophys. Acta* **255**, 331–347.
13. Bittman, R. & Blau, L. (1972) *Biochemistry* **11**, 4831–4839.
14. Schwenk, E. & Werthessen, N. T. (1952) *Arch. Biochem. Biophys.* **40**, 334–341.
15. Bleaney, B., Dobson, C. M., Levine, B. A., Martin, R. B., Williams, R. J. P. & Xavier, A. V. (1972) *J. Chem. Soc. Chem. Commun.* 791–793.
16. Rand, R. P. & Luzzati, V. (1968) *Biophys. J.* **8**, 125–137.
17. Levine, Y. K. & Wilkins, M. H. F. (1971) *Nature New Biol.* **230**, 69–72.
18. Trauble, H. & Haynes, D. H. (1971) *Chem. Phys. Lipids I*, 324–335.